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APPLICATION NUMBER: ***60/460,397***
FILING DATE: ***April 07, 2003***

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PTO/SB/16 (10-01)
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Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

Express Mail Label No.

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☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

PHARMACEUTICAL COMPOSITIONS AND METHODS FOR TREATING MULTIDRUG RESISTANT CANCER

CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 28 ☐ CD(s), Number [ ]

☒ Drawing(s) *Number of Sheets* 7 ☐ Other (specify)

☒ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☐ Applicant claims small entity status. See 37 CFR 1.27.

☒ A check or money order is enclosed to cover the filing fees

☐ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: [ ]

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Respectfully submitted,

SIGNATURE [signature]

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Date Jan 21, 2003

REGISTRATION NO. *(if appropriate)* [ ]

Docket Number: [ ]

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FEE TRANSMITTAL for FY 2003

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☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 80.00

Complete if Known	
Application Number	
Filing Date	
First Named Inventor	Stuart A. Berger
Examiner Name	
Art Unit	
Attorney Docket No.	

METHOD OF PAYMENT (check all that apply)

☑ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☐ Deposit Account:

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	750	2001	375	Utility filing fee	
1002	330	2002	165	Design filing fee	
1003	520	2003	260	Plant filing fee	
1004	750	2004	375	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80.00

SUBTOTAL (1) ($) 80.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

	Extra Claims		Fee from below	Fee Paid
Total Claims	- 20** =	X		0
Independent Claims	- 3** =	X		0
Multiple Dependent				

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	84	2201	42	Independent claims in excess of 3
1203	280	2203	140	Multiple dependent claim, if not paid
1204	84	2204	42	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0

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FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920	1804	920	Requesting publication of SIR prior to Examiner action	
1805	1,840	1805	1,840	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	410	2252	205	Extension for reply within second month	
1253	930	2253	465	Extension for reply within third month	
1254	1,450	2254	725	Extension for reply within fourth month	
1255	1,970	2255	985	Extension for reply within fifth month	
1401	320	2401	160	Notice of Appeal	
1402	320	2402	160	Filing a brief in support of an appeal	
1403	280	2403	140	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,300	2453	650	Petition to revive - unintentional	
1501	1,300	2501	650	Utility issue fee (or reissue)	
1502	470	2502	235	Design issue fee	
1503	630	2503	315	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment, per property (times number of properties)	
1809	750	2809	375	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	750	2810	375	For each additional invention to be examined (37 CFR 1.129(b))	
1801	750	2801	375	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify)

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($) 0

SUBMITTED BY

(Complete if applicable)

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60460397.040703

Stuart A. Berger
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January 21, 2003

The Commissioner of Patents
& Trademarks
Washington, D.C. 20231
U.S.A.

Dear Commissioner:

Re: New US Provisional Patent Application
Title: Pharmaceutical Compositions and Methods for Treating Multidrug Resistant Cancer
Inventors: Stuart A. Berger & Yicheng Zhang

Enclosed herewith please find the following documents regarding the above new US provisional patent application that is being filed today:

1) Provisional Patent Application Cover Sheet;
2) Application Data Sheet;
3) Fee Transmittal;
4) Cheque for $80.00 US; and
5) Provisional Patent Application.

Respectfully submitted,

Stuart A. Berger

Encl.

Initial Information Data Sheet

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Application Information

Title Line One::	Pharmaceutical Compositions
Title Line Two::	and Methods for Treating
Title Line Three::	Multidrug Resistant Cancer
Total Drawing Sheets::	7
Formal Drawings?::	n/a
Application Type::	Prov.
Docket Number::	

Representative Information

Representative Customer Number::

Continuity Information

This application is a::
> Application One::
Filing Date::

This application is a::
> Application Two::
Filing Date::

which is a::
>> Application Three::
Filing Date::
which is a::
>> Application Four::
Filing Date::

Prior Foreign Applications
Foreign Application One::
Filing Date::
Country::

Pharmaceutical Compositions and Methods for Treating Multidrug Resistant Cancer

Field of the Invention

The invention relates to pharmaceutical compositions and methods for chemotherapy. The invention reverses multidrug resistance to chemotheraputic agents and prevents cardiac damage caused by chemotheraputic agents.

Background of the Invention

Intrinsic or acquired resistance to chemotherapeutic agents is a major contributing factor to failure in cancer treatment. Clinical drug resistance often presents as a multi-drug resistance (MDR) phenotype, characterized as *de novo* resistance to a variety of structurally diverse cytotoxic drugs or as developed cross-resistance to chemotherapeutic agents that have never been used in previous chemotherapy [17]. Although the cellular basis underlying drug resistance is not fully understood, several factors have been identified that contribute to its development. These include drug efflux mechanisms, increased drug inactivation (e.g. glutathione-S-transferase and resistance to alkylating agents), drug target mutation (topoisomerase mutation), altered DNA repair and resistance to apoptosis (p53 mutation, bcl-2 overexpression etc.) [1]. Clinical drug resistance may be caused by any one or a combination of these mechanisms. Increased transmembrane efflux of xenobiotics is one of the best characterized mechanisms of MDR and is known to be mediated through over-expression of adenosine triphosphate (ATP)-binding cassette (ABC) transporter superfamily members such as P-glycoprotein (P-gp / MDR1), multidrug resistance associated protein (MRP1), or breast cancer

resistance protein (BCRP) [5, 14, 19, 20]. P-gp, the most extensively studied of these transporters, is encoded by the mdr1 gene and found to be overexpressed in many tumor cells that are resistant to anthracyclines or vinca alkaloids. Transfection of the mdr1 gene to drug-sensitive cell lines can transfer the MDR phenotype [28]. In about 30-40% of primary and more than 50% of metastatic breast cancer patient samples, P-gp was overexpressed [16, 27]. Increased expression of P-gp correlates with adverse prognosis and is associated with poor chemotherapy response and overall survival [27].

The prognostic importance of P-gp overexpression shows that the ability to prevent or reverse multi-drug resistance would be clinically valuable. This has led to the identification of a wide variety of compounds that are capable of reversing MDR through the inhibition of P-gp. Preclinical *in vitro* and *in vivo* studies in mice using MDR reversing agents such as verapamil, quinidine and cyclosporine A have demonstrated enhanced anti-MDR tumor activity [9]. To date, clinical trials have been conducted to evaluate the efficacy of MDR reversing agents with mixed results. In some cases, serum levels of reversing agents needed to block Pgp could not be achieved. In other cases, Pgp could be blocked but the levels of chemotherapeutic drugs had to be reduced in order to prevent excessive toxicity. However, some small scale studies in P-gp positive AML and VAD-refractory multiple myeloma showed that incorporation of verapamil or cyclosporin in chemotherapy significantly improved overall survival [6, 18, 26]. New, more potent P-gp inhibitors such as PSC388, GF120918, dexverapamil and XR9576 are also currently being evaluated in clinical trials and to date, preliminary results indicate that at minimum, it is possible to obtain serum levels of reversing agents sufficient to block P-gp [22, 24]. However, there remains a need for compositions that more effectively prevent multi-drug

resistance. As mdr1/P-gp is also expressed in certain normal tissues, blockade of P-gp *in vivo* by reversal drugs inevitably changes drug distribution and metabolism, thus altering the pharmacokinetics of chemotherapeutic agents. As a result, increased accumulation of the drugs in plasma or tissue can cause increased toxicity (Table 1).

TABLE 1: Cardiotoxicity of chemotherapy

Drug	Toxic dose range[a]	Toxicity
Amsacrine	Conventional dose	Ventricular arrhythmia
Busulfan	Oral daily dose	Endocardial fibrosis
Cisplatin	Conventional dose	Acute myocardial ischemia
Cyclophosphamide	> 100-120 mg/kg over 2 d	Congestive heart failure, hemorrhagic myocarditis/pericarditis/necrosis
Daunorubicin	> 550 mg/m^2 (total dose)	Same toxicity as doxorubicin
Doxorubicin	> 550 mg/m^2 (total dose)	Congestive heart failure (cumulative toxic effect), arrhythmias
	< 550 mg/m^2 (total dose)	Cardiac toxicity in the presence of additional risk factors
Fluorouracil	Conventional dose	Angina/myocardial infarction
Interferons	Conventional dose	Exacerbation of underlying cardiac disease
Interleukin-2	Conventional dose	Acute myocardial injury, ventricular arrhythmia, hypotension
Mitomycin	Conventional dose	Myocardial damage similar to radiation-induced injury
Mitoxantrone	> 100-140 mg/m^2 (total dose)	Congestive heart failure, decreased left-ventricular ejection fraction
Paclitaxel	Conventional dose	Bradycardia
Vinblastine	Conventional dose	Myocardial infarction
Vincristine	Conventional dose	Myocardial infarction

[a] Route of administration is IV unless otherwise indicated. Conventional dose is commonly accepted therapeutic range.

Adapted, with permission, from Grever MR, Grieshaber CK: Toxicology by organ system. In Holland JF et al (eds): Cancer Medicine, 4th ed, p 977. Baltimore, Williams & Wilkins, 1997.

Doxorubicin, one of the most potent chemotherapeutic agents for treating hematological malignancies and solid tumors, has dose-limiting cardiotoxicity both in animal models

and in cancer patients. In one study, Coadministration of cyclosporin and doxorubicin resulted in 55% and 350% increase of area-under-the-curve (AUC) of doxorubicin and its metabolite doxorubicinol respectively (Bartlett, 1994). PSC388, when used in combination with doxorubicin, increased doxorubicin AUC by 10 –fold [12]. Using a murine model, Sridhar showed that the combination of verapamil and doxorubicin increased peak doxorubicin concentration in heart tissue by about 40% compared to doxorubicin alone. This increased tissue doxorubicin level led to severe heart damage and significantly lower survival rate [25]. There is a need for compositions and methods for chemotherapy which do not cause heart damage. The need applies to the most common chemotherapy drugs, such as those shown in Table 2.

Table 2. Cytotoxic drugs which are transported by P-gp.

Anthracyclines	*Vinca* Alkaloids
Doxorubicin	Vincristine
Daunorubicin	Vinblastine
Idarubicin	Vinorelbine
Epirubicin	Others
Epipodophyllotoxings	Mitoxantrone
Etoposide	Dactinomycin
Teniposide	Amsacrine
Taxanes	Trimetrexate
Paclitaxel	Mitomycin
Docetaxel	Mithramycin

Summary of the Invention

The invention relates to a pharmaceutical composition comprising i) ketotifen or an analog thereof and ii) doxorubicin or an analog thereof. The pharmaceutical composition is useful for treating cancer. The pharmaceutical composition is also useful for i)

circumventing or treating multi-drug resistance in a subject or ii) preventing doxorubicin or doxorubicin analog induced cardiac tissue damage in a subject. The invention also includes kits containing these compositions and methods of use of these pharmaceutical compositions. In variations of the invention, mitoxantrone, VP-16 and vinblastine, or analogs thereof, are useful in the compositions and methods of the invention in place of doxorubicin. Other useful compounds which are subject to P-gp-mediated efflux (preferably those compounds in the same class that have similar activities as doxorubicin, mitoxantrone, VP-16 and vinblastine and which do not cause cause cardiotoxicity) are described below. We also use ketotifen and its analogs or compounds such as cetirizine and mizolastine with chemotherapy drugs described in this application (or analogs thereof).

The invention relates to a method for treating cancer, comprising stimulating cancer cells with a composition that is a Ca^{2+}-mobilizing agonist while concurrently blocking Ca^{2+} influx, whereby the cancer cells are sensitized to cell death induction. Ketotifen is a first generation antihistamine with store-operated Ca^{2+} channel antagonist properties [10]. As a calcium influx blocker, it was previously demonstrated that ketotifen could induce cell death in an activation-enhanced manner in leukemia cells [13], mast cells [23], and breast cancer cells [29]. In the course of evaluating the ability of ketotifen to induce cell death in breast cancer cells, it was observed that ketotifen could sensitize multi-drug resistant human breast cancer cells to doxorubicin. The invention shows that ketotifen can reverse multi-drug resistance through inhibition of P-gp. More importantly, it shows that ketotifen also reduces cardiotoxicity caused by high dose doxorubicin *in vivo* thus uniquely identifying ketotifen as both a MDR-reversing and cardioprotective agent.

Ketotifen restores sensitivity of P-glycoprotein-overexpressing, multi-drug resistant, MCF-7/adr cells to doxorubicin, mitoxantrone, VP-16 and vinblastine. *In vivo*, it was demonstrated that pretreatment of mice with ketotifen caused an increased accumulation of doxorubicin in cardiac tissue, consistent with a block in drug clearance. However, it was also observed that unlike verapamil, ketotifen pre-treatment did not enhance doxorubicin toxicity but in fact provided protection, both at the level of cardiac tissue damage and in survival. The invention provides the surprising invention that ketotifen reverses multi-drug resistance due to P-glycoprotein overexpression and provides cardioprotection to doxorubicin.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

Preferred embodiments of the invention will be described in relation to the drawings in which:

Fig.1 Dose response curve of ketotifen and verapamil as MDR reversal compounds. MCF-7/adr cells were treated with different concentrations of reversal compounds with or without 2μM doxorubicin for 24 hours. Cells were harvested , and plated for growth of breast cancer colonies in triplicates. The data are presented as perent of control clonogenicity in the absence of drug ($p < 0.0001$). DXN: doxorubicin.

Fig.2 Influence of 10μM of ketotifen on the toxicities of four cytotoxic drugs. MCF-7/adr cells were treated with different concentrations of chemotherapeutic agents in the presence or absence of 10μM ketotifen for 24 hours. Cells were harvested, washed and plated for growth of breast cancer colonies as described in the Materials and Methods. The data are presented as perent of control clonogenicity in the absence of drug ($p<0.0001$). Ke: ketotifen.

Fig.3 Ketotifen fails to reverse MDR of MCF-7/mx and MCF-7/vp cell lines. MCF-7/mx and MCF-7/vp cell lines were treated and assayed as described in Figs. 1 and 2. The toxicities of mitoxantrone and Vp-16 were evaluated respectively in MCF-7/mx and MCF-7/vp cells. Ke: ketotifen.

Fig.4 Flow Cytometric analysis of intracellular doxorubicin retention. MCF-7/adr cells ($5x10^5$/ml) were incubated with 2μg/ml of doxorubicin at 37°C for 2.5 hours in the presence of different concentrations of ketotifen or verapamil. Cells were washed and resuspended in ice-cold PBS. Doxorubicin relative fluorescence was measured by flow cytometry. A: 0 μM, b: 2 μM, c: 10 μM ketotifen or verapamil.

Fig.5 Doxorubicin accumulation in heart tissue. Mice were treated with i.p. injection of reveral agents ketotifen or verapamil, followed 30 minutes later by 15mg/kg doxorubicin or saline (as control). Three hours following injection of doxorubicin, three mice were sacrificed in each group and the hearts were excised, rinsed,minced and homogenated. The tissue doxorubicin was extractd with ice-cold acid ethanol solution (0.3N HCl in 50% ethanol). The doxorubicin in the supernatants was measured by

fluorescence spectrometry. DXN: doxorubicin, VPL: verapamil, Ke: ketotifen. **: p<0.01.

Fig.6 Histological evaluation of cardiotoxicity. Mice were injected with reversal compounds ketotifen (25mg/kg) or verapamil (25mg/kg) followed by doxorubicin (15mg/kg). Three mice in each group were sacrificed 4 days post treatment. The hearts were removed and fixed in 10% formalin. At least 3 sections were made and stained with H.E.. Slides were evaluated by light microscopy (original magnification = 400X). In mice receiving doxorubicin alone, some capillary dilation, degeneration and vacuolization can be readily observed. Combination of verapamil with doxorubicin aggravates these manifestations of cardiotoxicities especially the cytoplasmic vacuolization. Ketotifen, on the other hand, alleviates these pathological changes induced by doxorubicin.

Fig.7 Modulation of doxorubicin toxicity by verapamil or ketotifen. Mice were injected with reversal compounds ketotifen (25mg/kg) or verapamil (25mg/kg) followed by doxorubicin (15mg/kg). Mice were observed for survival for 30 days following treatment. ***: p<0.001

Detailed Description of Invention

The invention relates to a pharmaceutical composition comprising i) ketotifen or an analog thereof and ii) doxorubicin or an analog thereof. The pharmaceutical composition is useful for treating cancer. The pharmaceutical composition is also useful for i) circumventing or treating multi-drug resistance in a subject and ii) preventing doxorubicin or doxorubicin analog induced cardiac tissue damage in a subject. The anthracycline protective effects and reversal of MDR were unknown prior to this invention.

The invention also includes a kit comprising the agents i) ketotifen or an analog thereof and ii) doxorubicin, and directions for administering i) and ii) to a subject, preferably for

administering the agents to treat cancer, i) prevent or treating multi-drug resistance in a subject or ii) prevent doxorubicin or doxorubicin analog induced cardiac tissue damage in a subject.

The invention also includes a method for treating cancer in a subject, comprising administering to the subject the pharmaceutical composition of the invention or the agents of the kit of the invention. The cancer can be a solid tumor or a hematological malignancy.

The invention also includes a method for i) circumventing or treating multi-drug resistance in a subject or ii) preventing doxorubicin or doxorubicin analog induced cardiac tissue damage in a subject, comprising administering to the subject the pharmaceutical composition of claim 3.

A preferred embodiment of the invention includes a method for treating cancer in a subject, including

> administering to the subject an effective amount of ketotifen or an analog thereof, and
>
> administering to the subject an effective amount of doxorubicin or an analog thereof.

Preferably, the ketotifen is administered prior to the doxorubicin, more preferably at least 30 minutes prior to the doxorubicin. In the methods, the ketotifen or analog thereof and doxorubicin or analog thereof are preferably administered orally, intravenously, intraperitoneally, subcutaneously or rectally or by a combination of more than one of the foregoing.

Ketotifen

Ketotifen has the chemical name 4-(1-Methyl-4-piperidylidene)-4*H*-benzo[4,5]cyclohepta[1,2-*b*] thiophen-10(9*H*)-one hydrogen fumarate and the molecular formula $C_{23}H_{23}NO_5S$ (Chemical Abstracts Registry Number for Ketotifen is 34580-13-7). It is described, for example, in U.S. Pat. Nos. 3,682,930, 3,749,786, and 5,399,360. Examples of ketotifen analogs are found in U.S. Pat. Nos. 3,682,930 and 3,749,786. The

preferred ketotifen analogs are those which are suitable for use in mammals, such as humans. Methods which may be employed in screening and identifying useful ketotifen analogs and derivatives are described, for example, in U.S. Pat. No. 3,749,786.

Doxorubicin

Doxorubicin has the chemical name 8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy-a-L-*lyxo*-hexopyranosyl)oxy] -8-glycolyl-7,8,9,10-tetrahydro-6,8,11- trihydroxy-1-methoxy-5,12-naphthacenedione hydrochloride. The molecular formula of the drug is $C_{27}H_{29}NO_{11}$•HCl and its analogs are also known in the art. Analogs include mitoxantrone, daunorubicin. and N-acetyl daunorubicin. Other doxorubicin analogs are described in US Patent Nos. 4,672,057, 4,345,068, 4,314,054, 4,229,355, 4,216,157, 4,199,571, 4,138,480.

Other compounds are described below. Preferred compounds and analogs have at least 25%, 50%, more preferably at least 75% of the activity of doxorubicin and ketotifen for reversing MDR without cardiotoxicity. Activity may be measured by a MDR assay or cardioprotection study as described in this application.

"Preventing" or "Reversing" drug resistance means inhibiting P-gp to circumvent, reduce or avoid MDR. It does not necessarily mean modifying the cancer cells so that they no longer have the MDR phenotype of overexpressed P-gp.

Pharmaceutical Compositions

The above described substances may be formulated into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals. Compositions for chemotherapy are described in *Cancer Chemotherapy Handbook* by David S. Fischer, et al. (5th Ed., Mosby-Year Book, Inc. Publication date, May, 1997); *Lippincott's Cancer Chemotherapy Handbook* by Delia C. Baquiran, Jean Gallagher (Lippincott Williams and Wilkins Paperback); Physician's

Cancer Chemotherapy Drug Manual, 2002 and CD-ROM by Edward Chu, Vincent T. Devita (2002, Jones & Bartlett Pub)

Administration of a therapeutically active amount of pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the substance to elicit a desired response in the individual. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

An active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, rectal administration, inhalation, or transdermal application. Depending on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

The compositions described herein can be prepared by *per se* known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985) or Handbook of Pharmaceutical Additives (compiled by Michael and Irene Ash, Gower Publishing Limited, Aldershot, England (1995)). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and may be contained in buffered solutions with a suitable pH and/or be iso-osmotic with physiological fluids.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples. These

examples are described solely for the purpose of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances might suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

Ketotifen specifically reverses MDR mediated by P-pg transporter.

The toxicity of the cytotoxic drugs was measured by clonogenicity assay. As shown in Fig.1A, significant dose-dependent reversal of doxorubicin resistance was observed with ketotifen. Beginning at 1 μM, ketotifen restored doxorubicin toxicity while at 10 μM, the MDR phenotype of MCF-7/adr cells was completely reversed. Over this concentration range, ketotifen itself is non-toxic to MCF-7/adr cells. The ability of ketotifen to restore sensitivity of MCF-7/adr cells to doxorubicin was compared with verapamil. As shown in Figs. 1A and 1B, both ketotifen and verapamil reverse resistance at similar concentrations. MCF-7/adr cells are also relatively resistant to mitoxantrone, VP-16 and vinblastine. As shown in Fig.2, the sensitivity to these drugs was also restored by 10 μM ketotifen. The IC_{90}s of different cytotoxic drugs were calculated from dose-response curves for MCF-7/adr or MCF-7/wt cells in the presence or absence of 10 mM of ketotifen. As summarized in Table 3, IC_{90} levels on MCF-7/adr cells in the presence of ketotifen are almost identical to those for parental MCF-7 cells. In contrast to its reversing activity on MCF-7/adr cells, ketotifen influenced neither the toxicity of mitoxantrone on MCF-7/mx nor the toxicity of VP-16 on MCF-7/vp cells (Fig.3). These two cell lines exhibit the MDR phenotype by overexpressing BCRP [19] and MRP

transporters [21] respectively. Thus, ketotifen is a specific reversing agent for MDR associated with P-gp.

Table 3. IC_{90} (μM) of cytotoxic drugs on MCF-7/wt and MCF-7/adr

	MCF-7/wt	MCF-7/adr	
		-ke	+ke
Doxorubicin	0.12	>10	0.18
Mitoxantrone	0.01	0.22	0.02
VP-16	17	220	20
Vinblastine	0.8	410	1.2

Increased intracellular retention of doxorubicin in ketotifen treated MCF-7/adr cells.

Most MDR reversing agents act by inhibiting the transporting activity of P-gp. In order to determine if ketotifen inhibits P-gp activity, the intrinsic fluorescence of doxorubicin was used as a marker and measured drug accumulation by flow cytometry. MCF-7/adr cells pretreated with ketotifen or verapamil were exposed to doxorubicin and fluorescence was measured. As shown in Fig. 4, in the presence of either verapamil or ketotifen, fluorescence from doxorubicin increased in the pre-treated cells. 2 μM of ketotifen increased relative fluorescence by 50%, while 10 mM of ketotifen nearly doubled the fluorescence intensity. This result shows that ketotifen causes an accumulation of doxorubicin in MCF-7/adr cells and that ketotifen mediates its reversal ability through the inhibition of drug efflux.

Tissue doxorubicin concentrations in the heart.

To determine the interactions of ketotifen with cytotoxic drugs *in vivo*, mice were given i.p. injections of reversal agent, followed by 15 mg/kg doxorubicin. Tissue concentrations

of doxorubicin were determined by measuring doxorubicin fluorescence in heart tissue following different time periods after injection. The 3-hour time point values in different groups were compared as this point was the peak concentration was observed. As observed with verapamil, pre-treatment of mice with ketotifen significantly increased doxorubicin accumulation in the heart in comparison to control (72+/-5 vs 36 +/-3 ng/mg protein, $p<0.01$, Fig.5). This result shows that like verapamil, ketotifen causes a buildup of doxorubicin in tissue, likely due to inhibition of normal drug clearance mechanisms [25].

Ketotifen prevents cardiac tissue damage.

Cardiac tissue damage caused by anthracyclines is well known and characterized by cardiac hypertrophy, vacuolization disruption of myofibrils and cell loss [3]. In order to characterize the effect of combined MDR reversing agent plus doxorubicin treatment on heart tissue, mice were treated with ketotifen or verapamil followed by doxorubicin. Four days later, heart tissue was fixed, sectioned and stained with hematoxylin and eosin. As shown in Fig 6, mice treated with doxorubicin alone demonstrated well known pathological changes including dilation of capillaries, myocyte degeneration and vacuolization in left ventricular tissue (Fig. 6B). Addition of verapamil enhanced cardiac damage caused by doxorubicin (Fig.6C). In contrast, heart tissue from mice pre-treated with ketotifen (25mg/kg) 30 minutes before doxorubicin had observable decreases in the extent of cardiac damage (Fig.6D) with less cell drop-out, maintenance of myofibril structure and less vacuolization.

Cardiotoxicity and survival.

Since cardiac damage is reduced in mice receiving ketotifen plus doxorubicin, it was shown that the addition of ketotifen enhances mouse survival. Mice were pre-treated with ketotifen or verapamil, followed by a single treatment with doxorubicin and followed the animals over 5 weeks. Animals were sacrificed when they showed signs of lethargy or distress. As shown in Fig. 7, the survival rate of mice receiving doxorubicin plus verapamil was significantly lowered comparing to those mice treated with doxorubicin alone. For the doxorubicin plus verapamil group, survival rate at day 30 was 0% with, median survival time of 12.3 days while 42% of the doxorubicin alone group survived 30 days post treatment with a median survival of 19.3 days ($p<0.001$). In contrast, pre-treatment of mice with ketotifen led to extended survival compared to doxorubicin alone with 57% survival rate at day 30 and a median survival time of 23.2 days ($p<0.001$ compared to Dox + Verapamil). Since prolongation of survival in mice treated with ketotifen plus doxorubicin correlated with the protection of ketotifen on the cardiotoxicity induced by doxorubicin, these results clearly show that ketotifen enhances survival due to cardioprotection.

In this study, it was shown that the antihistamine ketotifen can reverse multi-drug resistance in MCF-7/adr cells through inhibition of P-gp. This effect is specific in that cells overexpressing BCRP or MRP are not affected by ketotifen. At high concentrations, ketotifen also blocks store-operated Ca^{2+} influx and induces activation enhanced cell death [13, 29]. However, ketotifen's P-gp-inhibitory activity appears to be unrelated to its Ca^{2+} channel blocking activity since the concentrations required for P-gp inhibition are much lower. Furthermore, it was observed that Ca^{2+} ionophores have no effect on the ability of ketotifen to reverse MDR (Zhang and Berger; unpublished), providing

additional evidence that ketotifen's MDR reversing activity is unrelated to its Ca^{2+} channel antagonism. It was further shown that pretreatment with ketotifen caused an increased accumulation of doxorubicin in mouse cardiac tissue, consistent with a block in drug clearance. However, unlike verapamil, ketotifen did not enhance doxorubicin toxicity but in fact provided protection, both at the level of cardiac tissue damage and in survival. These observations therefore show that ketotifen is unique in its ability to both reverse multi-drug resistance due to P-glycoprotein overexpression and provide cardioprotection to doxorubicin.

Although the mechanism of cardiotoxicity caused by anthracyclines is not fully understood, it is generally believed that highly active reactive oxygen species (ROS) triggered by anthracycine metabolites may play a central role in the initiation of a series reactions leading to myocyte damage [11, 15]. While antioxidants have shown some promise as cardioprotective agents *in vitro* and in animal models, clinical trials have not yet provided consistent benefit [4, 8]. Furthermore, the concern arises that the systemic application of antioxidants may also limit the anti-tumor efficacy of doxorubicin. Previous studies investigating the role of mast cell activation products in anthracycline-mediated cardiotoxicity had demonstrated that ketotifen could reduce doxorubicin cardiotoxicity and improve overall survival in a murine model [2]. In another study, doxorubicin was shown to induce mast cell degranulaton and histamine release, consistent with a role for mast cell activation in enhancing cardiac damage [7]. The invention shows the protective effect of ketotifen. Ketotifen's beneficial effect on survival can be partly attributed to cardiac protection based on the observed decrease in severity of cardiac damage in mice pre-treated with ketotifen.

The clinical use of anthracyclines is limited by its cardiotoxicity. Furthermore, schemes employing multi-drug reversing agents typically require reductions in chemotherapeutic dose due to inhibition of drug clearance mechanisms. The observations showing ketotifen as a multi-drug reversing agent with cardioprotective activity shows that this unique combination of properties is clinically useful in the control of multi-drug resistant tumors.

Materials and Methods

Human Breast Cancer Cell Lines and Culture Conditions.

MCF-7 (MCF-7/wt) and its multidrug resistant variant MCF-7/adr cells were used. MCF-7/mx and MCF-7/vp cell lines were also used. Other cell lines could also be used. MCF-7/mx cell line was generated through selection in vitro with mitoxantrone and overexpresses Breast Cancer Resistance Protein (BCRP) [19]. The MCF-7/vp cell line was selected with etoposide and overexpresses Multidrug Resistance-associated Protein gene (MRP) [21]. All the cell lines were grown routinely as monolayer culture in Dulbecco's Minimal Essential Media (DMEM) supplemented with L-glutamine (2mM), penicillin, streptomycin and 10% heat-inactivated fetal bovine serum (FBS, GIBCO) in an atmosphere of 5% CO_2 at 37°C. The cell lines were passaged weekly.

Chemicals.

Ketotifen, verapamil and all the chemotherapeutic agents (doxorubicin, etoposide VP-16, vinblastine and mitoxantrone) were purchased from Sigma Chemical Co. (St. Louis, MO). Ketotifen was freshly dissolved in DMSO before use, diluted with culture medium and added to the plate at the indicated concentrations. The final concentration of solvent

DMSO was always less than 0.1%. All the other drugs were dissolved either in DMSO (VP-16 and vinblastine) or saline and stored at -20°C as stock solutions.

Drug treatments and breast cancer clonogenic assay.

Exponentially growing MCF-7/wt and its three mutants MCF-7/adr, MCF-7/mx and MCF-7/vp were trypsinized, washed with fresh medium and plated in 6-well plates at a density of $1x10^5$/ml. Cytotoxic drugs of different concentrations were applied to cells in the presence or absence of ketotifen or verapamil for 24 hours. Both adherent and non-adherent cells were collected and washed with fresh medium. Cell aliquots ($5x10^3$) were plated in 1ml of 0.3% agar over 1 ml of of 0.5% agar underlayer prepared in IMDM containing 10% horse serum (GIBCO). The upper layer consisted of 20% FBS, 10 μg/ml of bovine insulin, 2.5 mg/ml of hydrocortisone, $5x10^{-7}$M of 17-b-estradiol (Sigma) and 50 ng/ml of EGF (R & D Systems). Colonies larger than 50 μm in size were scored after 14 days of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air.

Flow cytometry.

As doxorubicin itself is a fluorescent substance, the doxorubicin content in MCF-7/adr cells can be measured with flow cytometry. Briefly, cells ($5x10^5$/ml) were incubated with 2 μg/ml of doxorubicin at 37°C for 2.5 hours with or without reversal agents, washed and resuspended in ice-cold PBS. Doxorubicin fluorescence was measured by flow cytometry using a FACStar Plus flow cytometer (FL2, emission at > 570nm,Becton Dickinson). 10^4 cells were analysed for each sample.

Animals and in vivo treatment.

Female Balb/c mice (8-10 weeks of age, 20-22 g of body weight) were purchased from Jackson Laboratory (Maine, USA). Protocols were approved by the Animal Care Committee of the University Health Network. Animals were divided into 6 groups of 15 to 20 mice each and received drug treatments as follows: saline, ketotifen 25mg/kg, verapamil 25mg/kg, doxorubicin 15mg/kg plus saline, doxorubicin 15mg/kg plus ketotifen 25mg/kg, doxorubicin 15mg/kg plus verapamil 25 mg/kg. All the treatments were administrated via i.p. After treatment, mice were kept in sterile environment for six to eight weeks. Acute toxicities and survival was observed for different treatment groups. Mice were sacrificed when they displayed lethargic behaviour or any signs of distress. Three mice from each group were sacrificed on day 4- post treatment. Hearts were removed immediately and fixed in 10% neutral buffered formalin. Tissue sections were made from heart tissue and stained with hematoxylin and eosin or with 1% Toluidine blue to identify mast cells. All the slides were evaluated by light microscopy for cardiac damage, mast cell density and degranulation.

Doxorubicin concentrations in heart tissues.

3 to 5 mice in each group were treated with the same drug combinations used for survival. Three hours following injection of doxorubicin, mice were sacrificed. Doxorubicin concentrations in heart tissues were determined by flurometric detection of doxorubicin using the method of Sridhar [25]. Briefly, hearts were excised immediately, rinsed with ice cold normal saline, minced with scissors, and homogenized in ice cold ethanol- acid solution (0.3 N HCl in 50% ethanol) using a Polytron homogenizer. The homogenates were centrifuged at 20,000 g for 20 minutes at 4°C. Fluorescence of the supernatants was measured using a Tecan Spectrafluor (excitation wavelenth of 468,

emission wavelenth of 590, Hewlett Packard). The doxorubicin standard curve was made by mixing known amounts of doxorubicin with heart tissue and processed using an identical protocol. The fluorescence of supernatant from cardiac tissue without doxorubicin served as background. The concentration of doxorubicin was normalized to total protein content of the same tissue.

Statistical analysis.

All the colony data were analyzed by two-way analysis of variance (ANOVA), with differences between individual means determined by Bonferroni's post-tests. Data were expressed as means ± SEM. The Kaplan-Meier estimate was used to determine differences in the survival periods for mice following different drug combination treatments.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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We claim:

1. A pharmaceutical composition comprising i) ketotifen or an analog thereof and ii) doxorubicin or an analog thereof.

2. A pharmaceutical composition for use in treating cancer comprising i) ketotifen or an analog thereof and ii) doxorubicin or an analog thereof.

3. A pharmaceutical composition for use in i) preventing or treating multi-drug resistance in a subject or ii) preventing doxorubicin or doxorubicin analog induced cardiac tissue damage in a subject, comprising an effective amount of ketotifen or an analog thereof.

4. A kit comprising the agents i) ketotifen or an analog thereof and ii) doxorubicin, and directions for administering i) and ii) to a subject .

5. The pharmaceutical composition of claim 1 or 2 or the kit of claim 4, comprising the ketotifen, doxorubicin or analog thereof in an oral, intravenous, intraperitoneal, subcutaneal or rectal dosage form or in combination of the foregoing dosage forms.

6. A method for treating cancer in a subject, comprising administering to the subject the pharmaceutical composition of claim 1 or 2 or the agents of the kit of claim 4.

7. The method of claim 6, wherein the cancer comprises a solid tumor or a hematological malignancy.

8. A method for i) preventing or treating multi-drug resistance in a subject or ii) preventing doxorubicin or doxorubicin analog induced cardiac tissue damage in a subject, comprising administering to the subject the pharmaceutical composition of claim 3.

9. A method for treating cancer in a subject, comprising

administering to the subject an effective amount of ketotifen or an analog thereof, and

administering to the subject an effective amount of doxorubicin or an analog thereof.

10. The method of claim 9, wherein the ketotifen is administered prior to the doxorubicin.

11. The method of claim 10, wherein the ketotifen is administered at least 30 minutes prior to the doxorubicin.

12. The method of any one of claims 7 or 9 to 11 wherein the ketotofin or analog thereof and doxorubicin or analog thereof are administered orally, intravenously, intraperitoneally, subcutaneally or rectally or by a combination of more than one of the foregoing.

13. The use of the pharmaceutical composition of claim 1 or 2 or the agents of the kit of claim 4 for treating cancer in a subject.

14. The use of the pharmaceutical composition of claim 3 for i) preventing or treating multi-drug resistance in a subject or ii) preventing doxorubicin or doxorubicin analog induced cardiac tissue damage in a subject in a subject.

15. The use of ketotifen and doxorubicin for preparation of a medicament for treatment of cancer.

16. The use of claim 13 or 15, wherein the cancer comprises a solid tumor or a hematological malignancy.

17. A method of determing whether cancer should be treated with ketotifen or an analog thereof and doxorubicin or an analog thereof, comprising determining whether P-gp is overexpressed by the cell.

18. The method of claim 17, wherein the cancer comprises a solid tumor or a hematological malignancy.

19. The method of claim 17, further comprising treating the cancer with ketotifen or an analog thereof and doxorubicin or an analog thereof.

20. A method for i) preventing or treating multi-drug resistance in a subject or ii) preventing doxorubicin or doxorubicin analog induced cardiac tissue damage in a subject, comprising administering to the subject a compound having MDR-reversing

and anthracycline-protective activity.

Abstract

The invention relates to a pharmaceutical composition comprising i) ketotifen or an analog thereof and ii) doxorubicin or an analog thereof. The pharmaceutical composition is useful for treating cancer. The pharmaceutical composition is also useful for i) preventing or treating multi-drug resistance in a subject or ii) preventing doxorubicin or doxorubicin analog induced cardiac tissue damage in a subject.

Figure 1

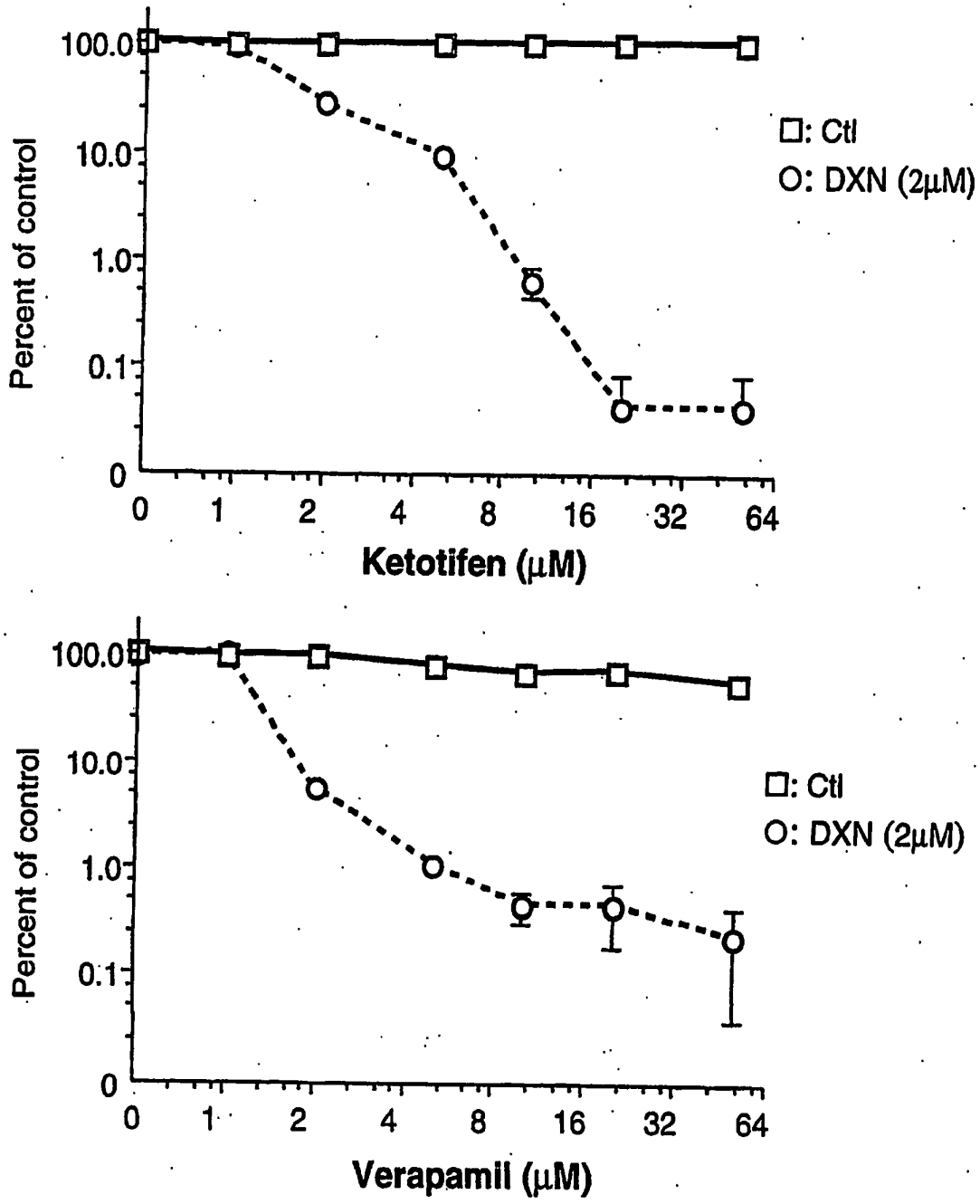
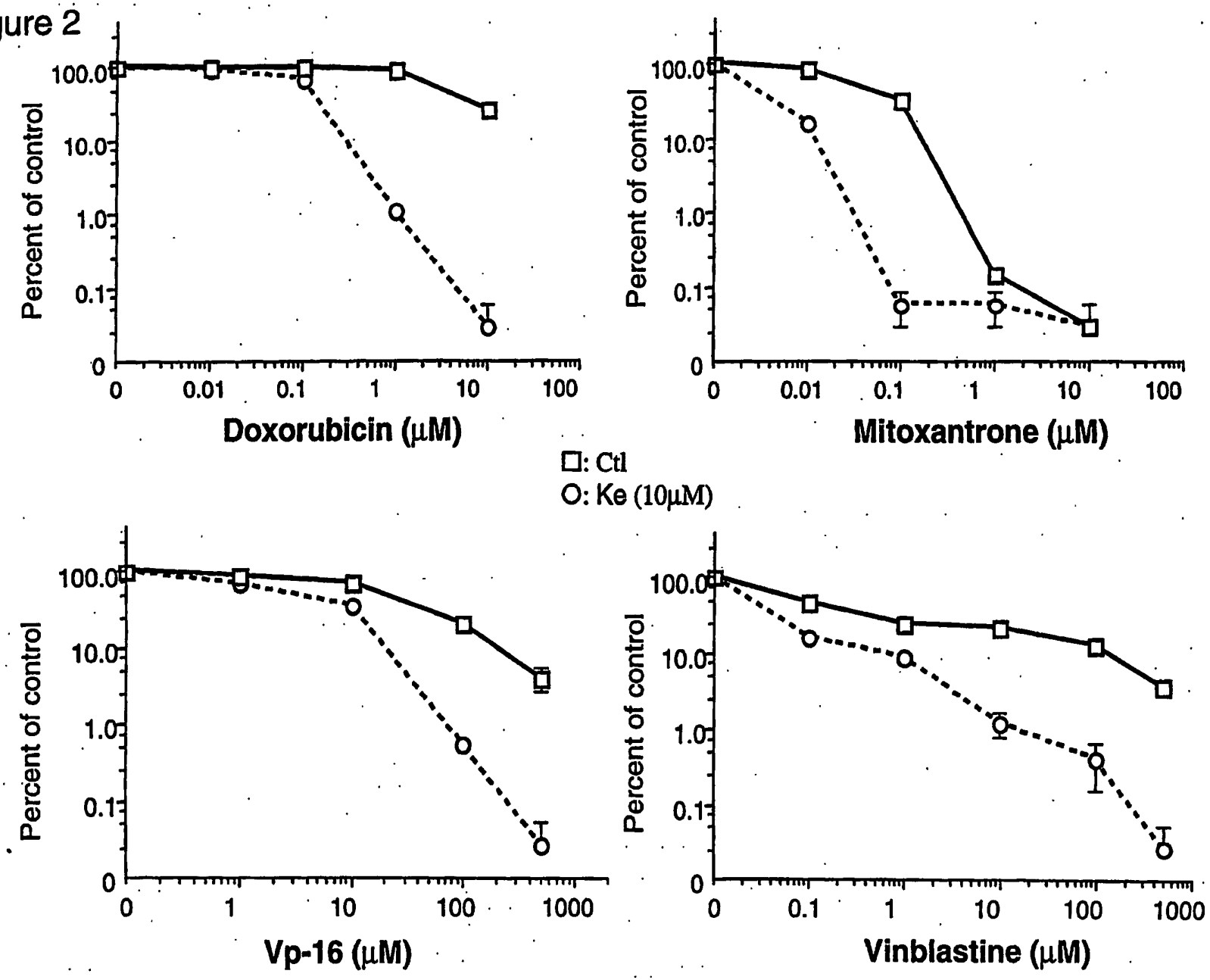

100.0
10.0
1.0
0.1
0
Percent of control
0
1
2
4
8
16
32
64
Ketotifen (μM)
□: Ctl
○: DXN (2μM)
100.0
10.0
1.0
0.1
0
Percent of control
0
1
2
4
8
16
32
64
Verapamil (μM)
□: Ctl
○: DXN (2μM)

Figure 2


Percent of control
100.0
10.0
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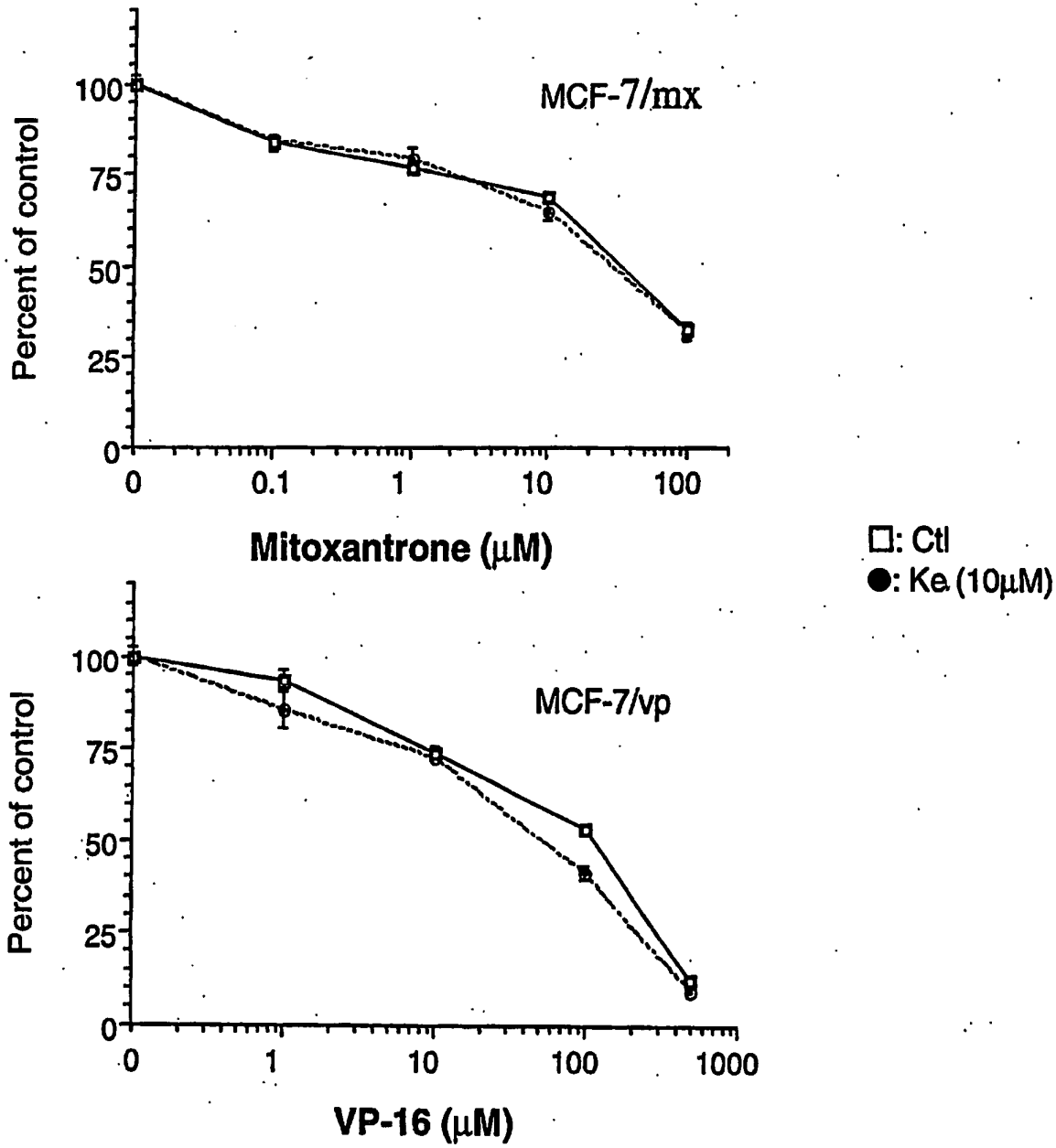
0.1
0
0.01
0.1
1
10
100
Doxorubicin (μM)
Mitoxantrone (μM)
□: Ctl
○: Ke (10μM)
1000
Vp-16 (μM)
Vinblastine (μM)

Figure 3


MCF-7/mx
Percent of control
100
75
50
25
0
0
0.1
1
10
100
Mitoxantrone (μM)
□: Ctl
●: Ke (10μM)
MCF-7/vp
Percent of control
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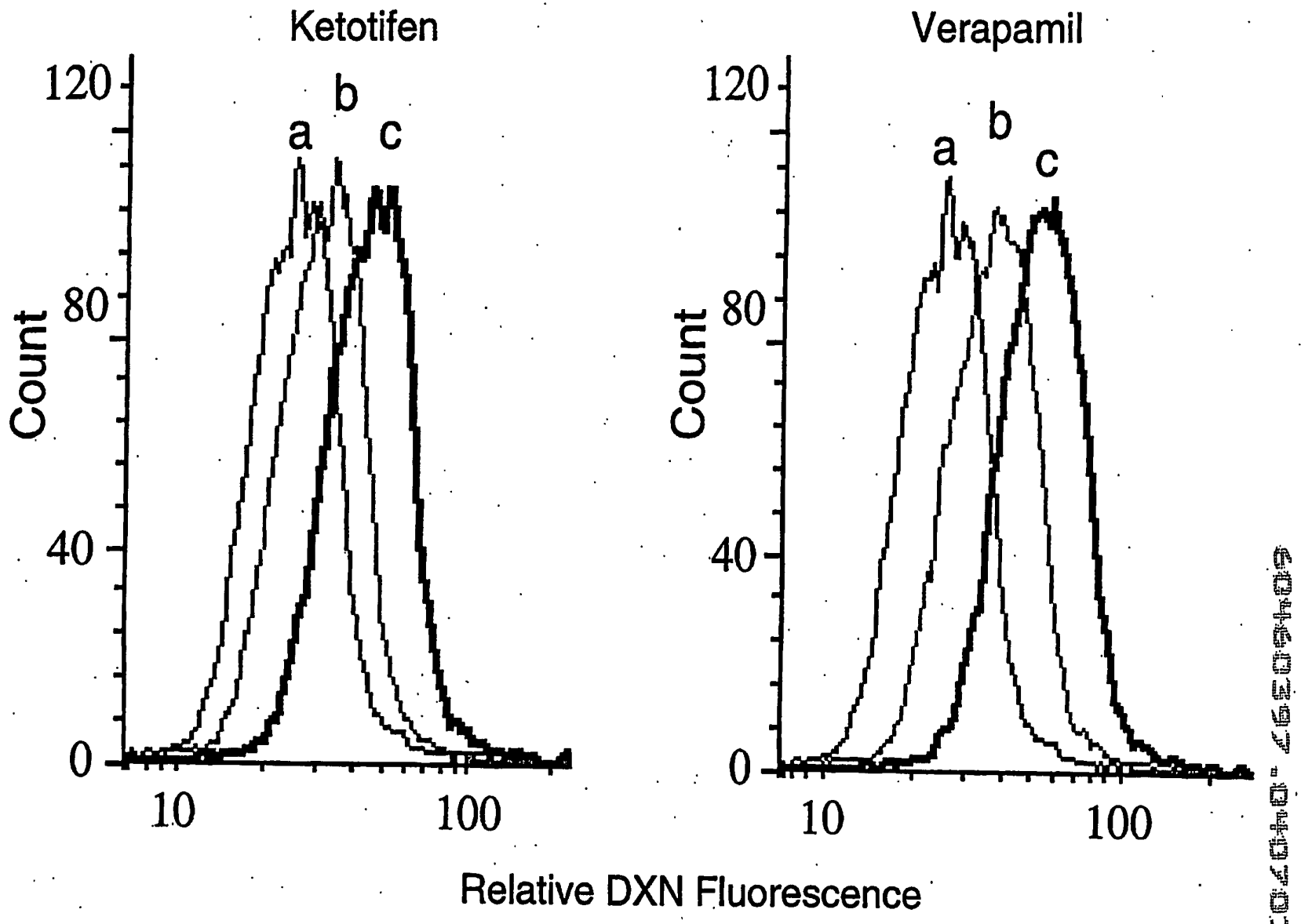
75
50
25
0
0
1
10
100
1000
VP-16 (μM)

Figure 4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Verapamil
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80
40
0
Count
a
b
c
10
100
Relative DXN Fluorescence

Figure 5

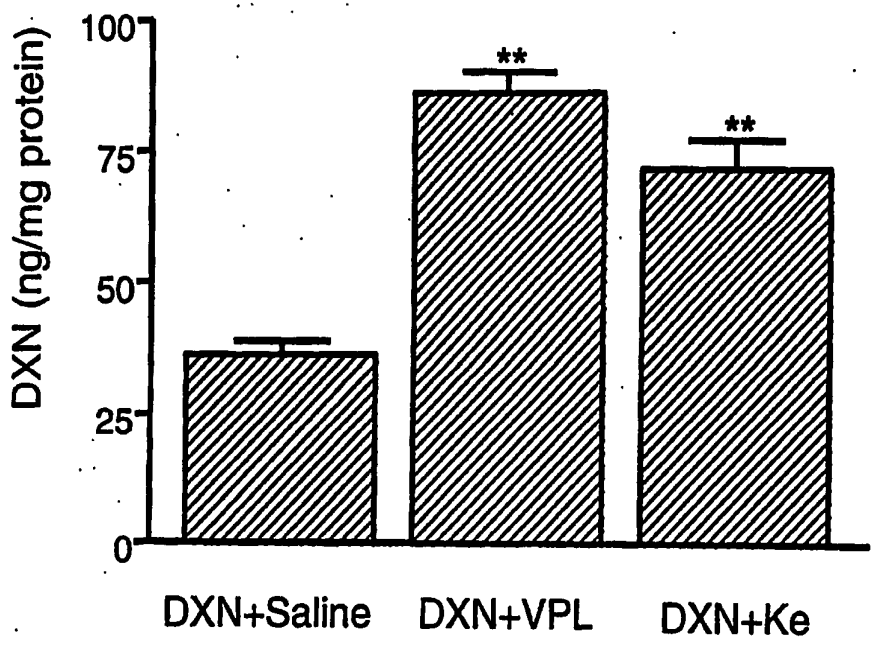

Figure 6

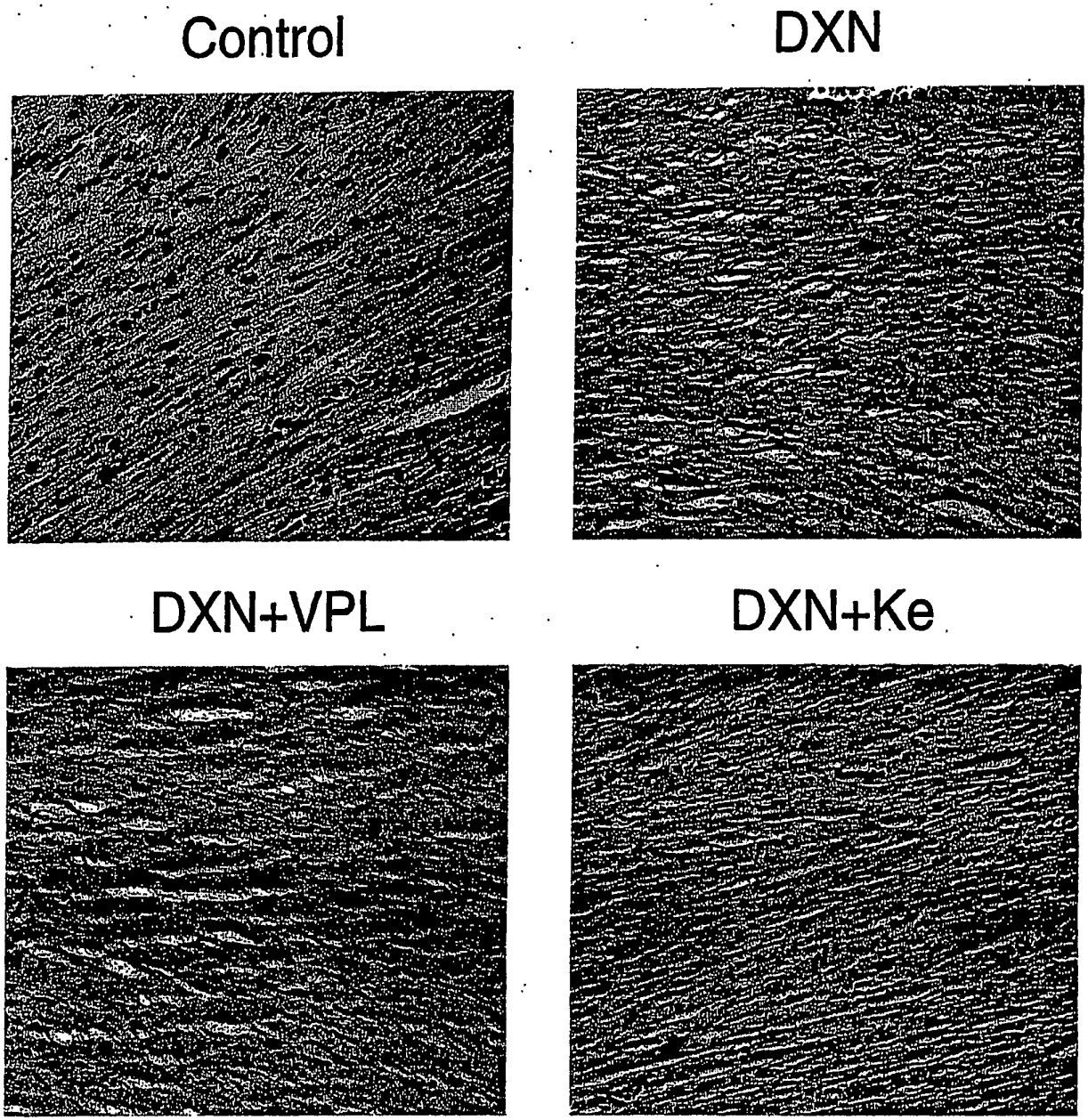

Figure 7